MECHANISMS IN PROTEIN SYNTHESIS. VI: A METHOD FOR MEASURING FAST KINETICS OF BINDING OF mRNA AND AMINOACYL-tRNA TO RIBOSOMES

Gernot SANDER and Heinrich MATTHAEI

Max-Planck-Institut für experimentelle Medizin, Göttingen, Germany

Received 13 February 1969

1. Introduction

A systematic study of the Mg^{2+}-dependence of individual reactions in the synthesis of polyphenylalanine in a cell-free system from *E. coli* was done recently [1]. The maximal initial velocity of the overall synthesis after a 2–3 min lag period (optimal conditions, 33°C, 0.75 mg/ml tRNA) was found to be around 1 $\mu\mu$mole phenylalanine/$\mu\mu$mole 70S ribosomes in 1 min. To investigate corresponding velocities in binding of aminoacyl-tRNA, a new method, faster than conventional techniques, had to be developed. We first applied it to the study of two non-enzymatic processes: (1) the binding of poly U, and (2) the binding of Phe-tRNA directed by poly U.

In this communication we present evidence that – at least at a reduced concentration of monovalent cations – the initial rate of non-enzymatic binding of poly U and Phe-tRNA to ribosomes would be high enough to account for the observed maximal polymerization velocity during chain elongation. This was shown by a modified cellulose-nitrate-filter technique [2] which has so far proved useful for measurements over periods of 20 msec to about 30 sec. A major part of this report will deal with the new experimental procedure. A few results obtained with it have been reported elsewhere [3,4].

2. Materials

Cells were grown and harvested [5], and ribosomes were prepared and washed 3 times with 0.5 M NH_4Cl in standard buffer as described previously [6,7].

^{14}C-labelled poly U * (spec. act. ~ 1 c/mole; average chain length = 150 nucleotides) was prepared with polynucleotide phosphorylase (step III of Matthaei et al. [5]). Unlabelled *poly U* was purchased from Miles Chem. Comp. For the preparation of *^{3}H-Phe-tRNA*, tRNA from *E. coli* B (Schwarz BioResearch) was charged with ^{3}H-phenylalanine (spec. act. 3000 c/mole) according to Heller [8].

"Detergent I" (C_{12}-C_{14}-alcohol, ethoxylated with 2 moles ethylene oxide and sulphated sodium salt) and other detergents tested were a gift from Henkel-Werke (Düsseldorf, Germany). Its molecular weight was assumed to be 390. *Filters:* type AAWP 25 mm diameter, from Millipore.

3. Method for kinetic measurements

3.1. *Principle*

Ribosomes are bound in the interior of Millipore filters by weak chemical interaction(s), thus allowing the layering of a poly U containing solution onto the filter without causing instantaneous reaction with the pre-bound ribosomes. Reaction times are determined from the volume of the solution applied and the rate of flow (see below). The apparatus used is described in fig. 1. Three bottles, held at different low pressures **, enable the adjustment of the vacuum to three defined values within a few seconds; fine setting is done with cocks 5 and 6.

* Poly U concentrations are expressed in terms of UMP, except where noted.

** Atmospheric pressure taken as zero.

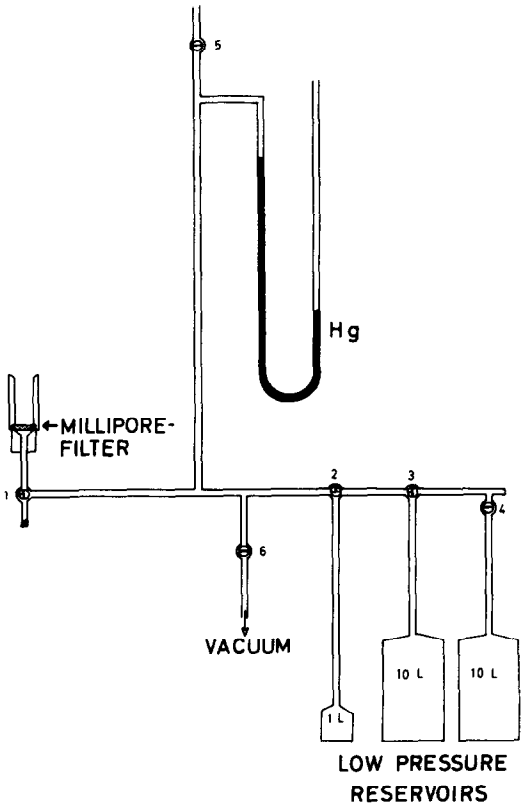

Fig. 1. Schematic illustration of apparatus for kinetic measurements using Millipore filters.

The Millipore filter type AAWP with a pore size of 0.8 μ being slightly larger than usually used was chosen for higher flow rates. It still gives good sorption of ribosomes. An almost linear relationship exists between pressure difference and flow rate. To correct for individual variations in the filters, each disc was calibrated at −60 mm Hg with 10 ml of *buffer I* (10 mM K-phosphate − 12 mM $MgCl_2$ − 0.01 mM detergent I).

3.2. *Binding of ^{14}C-poly-U to ribosomes*

After washing filter with 10 ml buffer I (calibration), the filter was charged with 50 $\mu\mu$moles ribosomes * (0°C) dissolved in 1 ml buffer I (20°C) at −5 mm Hg. All following steps were performed at 20°C. The filter was washed with 10 ml buffer I at −60 mm Hg. With cock 1 connecting filter and outlet, the rubber stopper closing the outlet was loosened and pushed upward again to introduce a slight rise in pressure underneath the filter, thus preventing the poly U solution from entering the filter. Cock 2 was opened and the vacuum adjusted to −600 mm Hg. The respective amount of poly U solution in buffer I was carefully placed on the filter, and cock 1 was turned at once to connect filter and low pressure reservoir. Immediately after the solution had passed through, the filter was washed with 20 ml buffer I, dried under an infrared lamp and counted in 5 ml of toluene − 0.4% PPO − 0.01% POPOP using a Tricarb liquid scintillation spectrometer (Packard).

3.3. *Poly U-dependent binding of ^{3}H-Phe-tRNA to ribosomes*

The low pressure was maintained at approximately −60 mm Hg to give an average flow rate of 1 ml/sec. All steps were performed at 20°C. Filters were calibrated as above, except that buffer I was replaced by *buffer II* (15 $MgCl_2$ − 10 mM K-phosphate pH 6.0). 50 $\mu\mu$moles of ribosomes * were incubated for 2 min with 30 mμmoles poly U in 100 μl reaction mixtures containing 20 mM $MgCl_2$ − 10 mM K-phosphate pH 6.0 − 6 mM KCl. After incubation, the reaction mixtures were diluted to about 4 ml and filtered. The filter with the bound poly U ribosome complex was washed 5 times with 4 ml buffer II. After raising the pressure slightly, as above, and adjusting the low pressure to −60 mm Hg, the volume of Phe-tRNA solution in buffer II required for a certain time of exposure was placed on the filter, and cock 1 was turned immediately to connect with low pressure. Thereupon the filter was washed with 20 ml buffer II. Dilution of Phe-tRNA with buffer II was done 20 min prior to each filtration. This preincubation, as well as the relatively low pH and high Mg^{2+} concentration, helps to avoid high blanks and increases by 100% the absolute amounts of Phe-tRNA bound. Detergents used to reduce filter-blanks in binding studies with ^{14}C-poly U were omitted, since they did not seem to have any influence on the binding of Phe-tRNA to filter or ribosomes in the presence or absence of poly U.

4. Results

Effect of detergent I on the binding of ^{14}C-poly U to ribosomes and filters is shown in fig. 2. The high minus-ribosome blank observed in the absence of

* Calculated at 16 A_{260} = 1 mg = 0.36 mμmoles ribosomes.

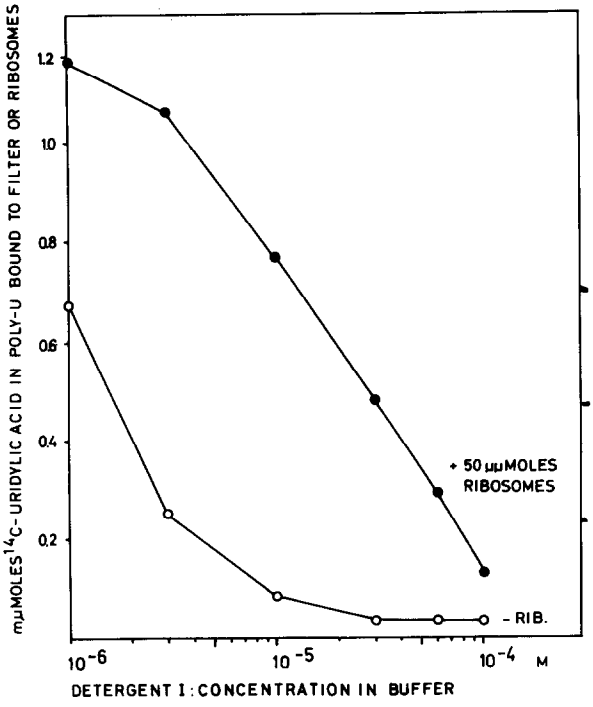

Fig. 2. Influence of detergent I upon binding of ^{14}C-poly U to Millipore filters without (= background) and with ribosomes.

detergent can be decreased to less than 10% of the full value by the employment of 10^{-5} M detergent I. This substance turned out to be especially suitable for experiments with poly U for two reasons: (1) it does not impede binding of ribosomes to the filters up to a concentration of 10^{-4} M; (2) its action is strictly concentration-dependent, i.e., neither pre-washing the filter, nor subsequent washing with 2–100 ml buffer containing 10^{-5} M detergent I produced any decrease in the binding of poly U. It should be noted that with shorter poly U chains (20–40 nucleotides), the use of detergents is less important.

The kinetics of the poly U binding reaction at six different poly U concentrations is shown in fig. 3a. Minus-ribosome blanks (in this case < 5%) are subtracted. The inclinations of at least the first three slopes completed in some of these kinetic curves are directly proportional to the poly U concentrations applied (see fig. 3b). In the first slope, 25–30% of the poly U offered is bound. Furthermore, the uppermost curve in fig. 3a shows that about one fourth of the maximal binding (50 μμmoles poly U chains per 50 μμmoles ribosomes [9]) can be accomplished within the first half second under appropriate conditions. In the experiment reported, 2.1 mμmoles ^{14}C-poly U

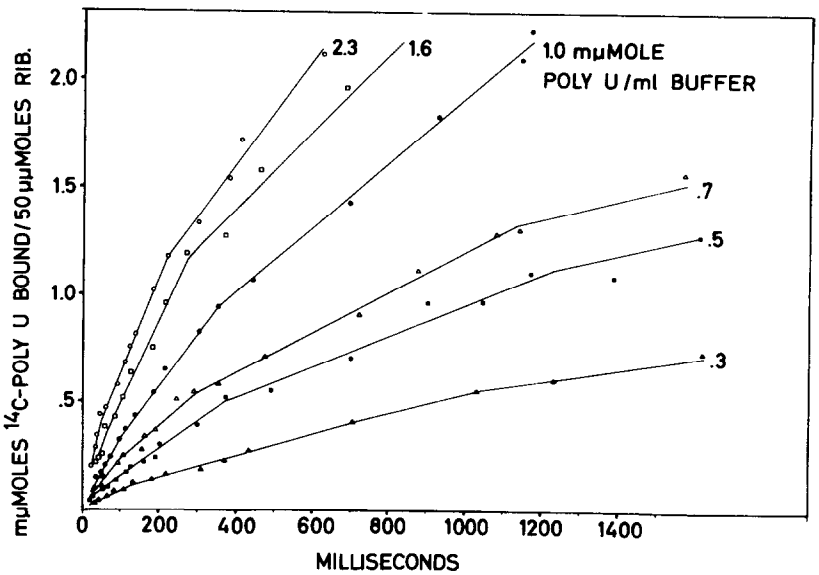

Fig. 3a. Binding of ^{14}C-poly U to ribosomes: kinetics at 20°C. Ribosomes prebound to Millipore filters (see Method); chain length approximately 150 nucleotide units; flow rate approximately 8 ml/sec; 50 μμmoles ribosomes.

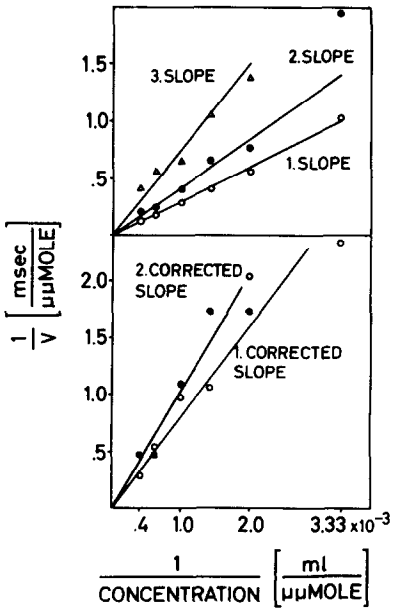

Fig. 3b. Relationship between concentration and initial velocity of the curves of fig. 3a. Correction is done by subtraction of superimposed slopes.

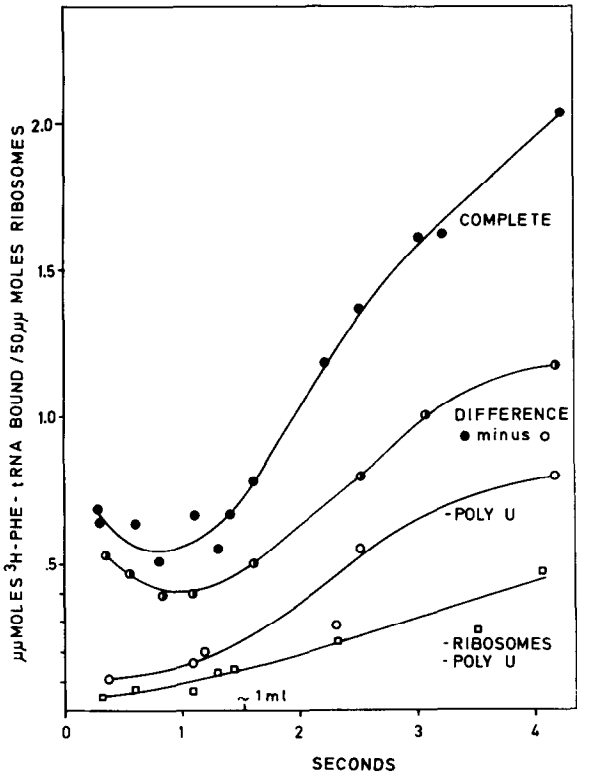

Fig. 4. Initial rate of binding of Phe-tRNA to ribosomes at 20°C. Ribosomes were saturated with poly U while in solution and bound to Millipore filters; thereupon, Phe-tRNA solution was applied (see Method); 180 μμmoles/ml ^{3}H-Phe-tRNA (= 6875 μμmoles total tRNA, calculated at 21 A_{260} units (measured in 10 mM KCl) = 1 mg; M.W. 25,000) in buffer II.

correspond to 14 μμmoles poly U chains.

From several of the kinetic curves in fig. 3a, it appears that these have at least 3 different slopes. Similarly, saturation curves taken for the binding of poly U to ribosomes at equilibrium (after 2 min at 20°C) have reproducibly displayed 4 straight slopes of decreasing steepness [9].

Binding of Phe-tRNA to ribosomes pre-saturated with poly U and bound to Millipore filters is illustrated in fig. 4. Curves of this type exhibited essentially the same shape, starting with an oscillatory phase and continuing in a long slope of decreasing steepness. The exact shape of the initial phase is difficult to reproduce. If we neglect the first 1.5 sec and regard the rate of binding between 1.5 and 3 sec as reflecting a true initial rate, we obtain 0.38 μμmoles Phe-tRNA bound/μμmole ribosomes in 1 min.

5. Discussion

Various individual component reactions in the initiation and elongation phases of polypeptide synthesis require the participation of at least the 3 initiation factors A, B and C, the factors G and T, and GTP [10]. Binding to ribosomes of poly U and Phe-tRNA, however, can be observed in the absence of such factors and GTP. These bindings are called "non-enzymatic". The binding of poly U to ribosomes reaches equilibrium in 2 min, whereas that of Phe-tRNA does so only after 60 min at room temperature. Correspondingly, the fraction of the offered substrate which is bound in the case of poly U appears to be one fourth during the first slope of the kinetic curve. For Phe-tRNA, however, it amounts to only 1/300. The initial oscillation(s) observed in the latter case may be ascribed to slow conformational transitions within the ribosome population. It would be premature to suggest an explanation for the three or more slopes observed in the kinetics of poly U binding.

Likewise, any causal connection between the four-sloped saturation curves (equilibrium) and the possible occurrence of four slopes in the kinetic curves should not be drawn, since the former were done with ribosomes in suspension and the latter with ribosomes attached to the filter. At 150 mg/ml tRNA, 0.2 $\mu\mu$moles phenylalanine would be incorporated per $\mu\mu$mole ribosomes in 1 min. The observed speed of 0.38 $\mu\mu$moles Phe-tRNA bound per $\mu\mu$mole ribosomes in 1 min at 20°C leaves a slight excess. This velocity must still be corrected for the higher temperature of 33°C, at which polyphenylalanine was synthesized. This rather satisfactory result is obtained, however, by comparison of speeds in different ionic environments: peptide synthesis in "high salt" (see ref. [1]), Phe-tRNA binding at lower ionic strength (see Method). The corresponding speeds for binding in "high salt" were considerably lower – in the range of 0.04 $\mu\mu$moles Phe-tRNA per $\mu\mu$mole ribosomes in 1 min. This result may indicate that some factor, acting in the enzyme-controlled elongation cycle [10], accelerates the binding of substrate. T-factor engaged in "amino-acyl-checking" [10] may be responsible here (cf. refs. [11–13]).

Compared to conventional methods in enzymology, the method described in this communication gives better resolution in time by three orders of magnitude. Even without further possible gain in resolution, it seems worthwhile as demonstrated in the case of ribosomal binding reactions.

References

[1] K.Løve, Hoppe-Seyler's Z. Physiol. Chem., in preparation.
[2] M.Nirenberg and P.Leder, Science 145 (1964) 1399.
[3] H.Matthaei, K.Løve, M.Milberg, G.Sander, D.Swan and H.-P.Voigt, in: Molecular Genetics (Springer-Verlag, Berlin, Heidelberg, New York, 1968) p. 81.
[4] J.H.Matthaei, H.-P.Voigt, G.Heller, R.Neth, G.Schöch, H.Kübler, F.Amelunxen, G.Sander and A.Parmeggiani, Cold Spring Harbor Symp. Quant. Biol. 31 (1966) 25.
[5] H.Matthaei, G.Heller, H.-P.Voigt, R.Neth, G.Schöch and H.Kübler, in: Genetic Elements, Properties and Function (Academic Press, New York and Polish Scientific Publishers, Warsaw, 1966) p. 233.
[6] H.Matthaei and M.W.Nirenberg, Proc. Natl. Acad. Sci. U.S. 47 (1961) 1580.
[7] H.-P.Voigt and H.Matthaei, Hoppe-Seyler's Z. Physiol. Chem. 349 (1968) 54.
[8] G.Heller, Ph.D. thesis, Göttingen (1966).
[9] G.Sander, Ph.D. thesis, Göttingen (1968); European J. Biochem., in preparation.
[10] H.Matthaei, G.Sander, D.Swan, T.Kreuzer, H.Caffier and A.Parmeggiani, Naturwissensch. 55 (1968) 281.
[11] F.Ibuki and K.Moldave, J. Biol. Chem. 243 (1968) 791.
[12] J.Shaeffer, R.Arlinghaus and R.Schweet, Arch. Biochem. Biophys. 125 (1968) 614.
[13] A.Skoultchi, Y.Ono, H.M.Moon and P.Lengyel, Proc. Natl. Acad. Sci. U.S. 60 (1968) 675.